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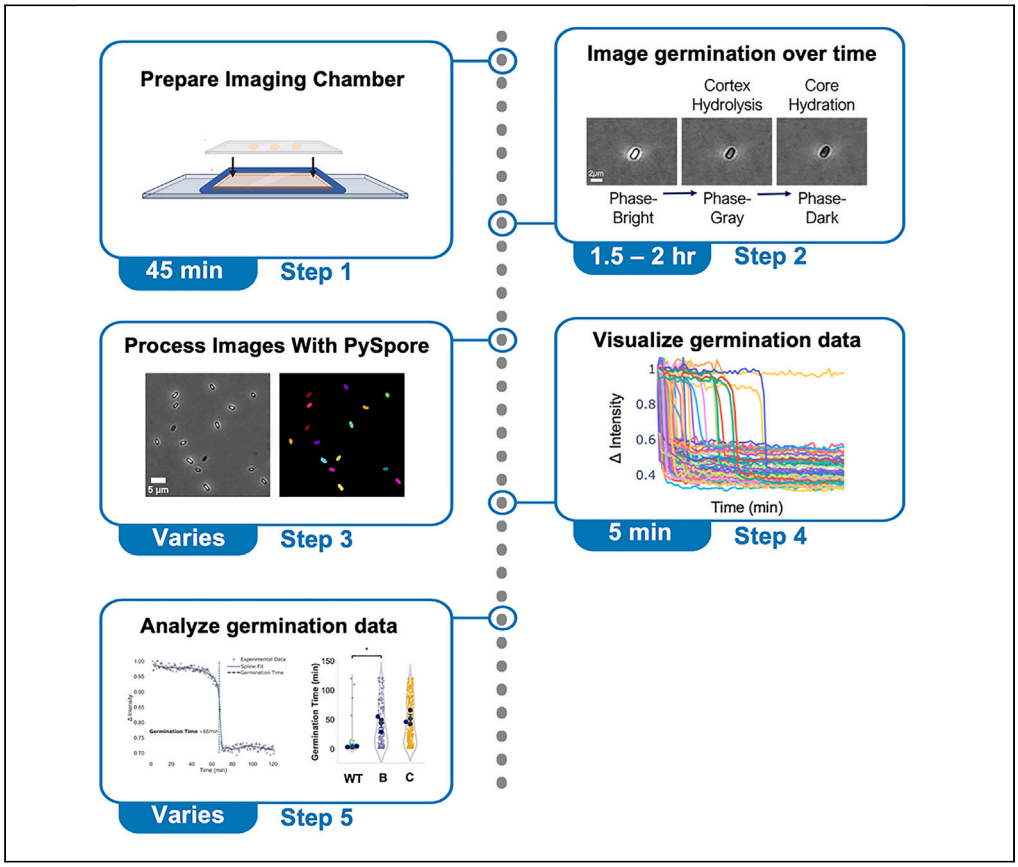
Protocol for quantifying the germination properties of individual bacterial endospores using PySpore



PySpore is a Python program that tracks the germination of individual bacterial endospores. Here, we present a protocol for segmenting spores and quantifying the germination properties of individual bacterial endospores using PySpore. We describe steps for using GUI-based tools to optimize image processing, annotating data, setting gates, and joining datasets for downstream analyses. We then describe procedures for plotting functionality tools without the user needing to modify the underlying code.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Custom image analysis pipeline for analyzing germination at the single-spore level

More accurate segmentation of spores than previously developed programs

Tools for optimizing image processing, joining datasets, and annotating data

GUI-based tools facilitate the visualization of germination data

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Protocol for quantifying the germination properties of individual bacterial endospores using PySpore

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SUMMARY

PySpore is a Python program that tracks the germination of individual bacterial endospores. Here, we present a protocol for segmenting spores and quantifying the germination properties of individual bacterial endospores using PySpore. We describe steps for using GUI-based tools to optimize image processing, annotating data, setting gates, and joining datasets for downstream analyses. We then describe procedures for plotting functionality tools without the user needing to modify the underlying code.

For complete details on the use and execution of this protocol, please refer to Ribis et al. (2023).[1]

BEFORE YOU BEGIN

Bacterial endospores are highly metabolically dormant forms of life that can be revived through the process of germination and outgrowth. Spore germination refers to the loss of spore resistance properties that occurs when the spore core/cytosol becomes more hydrated. Spore germination has historically been quantified using bulk methods that use optical density to measure the change in refractility that occurs when spores germinate.[2] This change in refractility ("phase-brightness") correlates with the degradation of the thick, protective peptidoglycan layer known as the cortex and rehydration of the partially dehydrated spore core (cytosol)[3] (Figure 1). While these methods have been in use for decades, they fail to capture the heterogeneity in germination that occurs between individual spores within a population.[4] Here, we present PySpore – a simple GUI-based program written in Python that quantifies the germination of individual spores from time-lapse phase-contrast microscopy images using a simple, easy-to-use interface. While current solutions exist,[5] PySpore is significantly easier to use and provides convenient GUIs for image processing, data processing, and basic data visualization. In addition, we provide a Jupyter Notebook from the accompanying manuscript that allows users to conduct more complex data analyses, including functions for quantification of germination rates and time to germinate. Furthermore, there are a series of classes and standalone functions found in the associated pyspore_utils.py file that allow users to customize the pipeline by exploiting the modularity of the program.

We have used this protocol to analyze the germination of *Clostridioides difficile* spores from different mutant backgrounds and in different conditions,[1] such as in the presence of different co-germinants,[6,7] buffer conditions, and germinant and co-germinant concentrations. However, we have also had success analyzing movies of germinating *Bacillus subtilis* spores (from the SporeTracker example data[5]) and anticipate that the program will be generalizable to phase-contrast images of spores from other endospore-forming bacterial species given their shared morphology.[8]

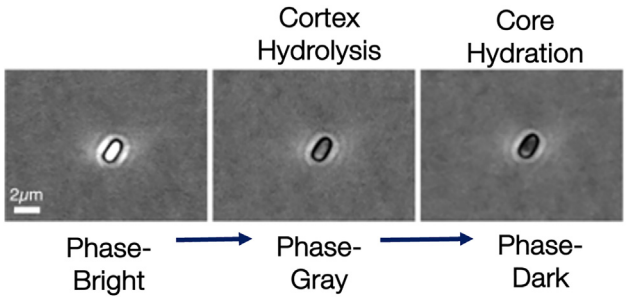

Figure 1. Spore germination analyses by phase-contrast microscopy

During germination, the refractility of dormant spores visualized by light microscopy changes as they become more hydrated. Hydration of the spore core occurs when the protective cortex layer is degraded during germination. Phase-gray spores have initiated germination and are becoming more hydrated as the cortex layer is being degraded. Phase-bright spores have completed germination because their cortex layer has fully degraded, resulting in full hydration of the core. Scale bar shows 2 μm.

While we have aimed to make this tool as accessible as possible to researchers, the minimum hardware requirements listed below should be noted before attempting this method.

1. Minimum computing hardware:
   a. 4-core CPU and 8 GB (preferably 16 GB) of RAM.

   Note: PySpore utilizes parallel CPU processing, and, as a result, more CPU cores will result in better performance.

2. Microscopy Hardware:
   a. 60–100× high numerical aperture oil immersion phase-contrast objective. DIC or brightfield images are not suitable for these analyses.
   b. Hardware auto-focus (e.g., Nikon Perfect Focus System, Zeiss Definite Focus, Leica Adaptive Focus Control). This ensures stable focus at all positions throughout the duration of the experiment. We have not tested software-based autofocus, but as long as it provides images that are in focus and drift between timepoints is within range, autofocusing should generate movies that are suitable for these analyses.
   c. Motorized, high precision stage.
   d. Software capable of time-lapse, multi-position imaging.

   Optional: Temperature-controlled enclosure (heating to 37°C increases the speed of germination).[9]

3. Download Required Software and Packages:
   a. Download the Anaconda Python distribution https://www.anaconda.com/products/distribution.
   b. Download the .zip folder containing code from https://github.com/jribis89/PySpore and extract all files to your desired location (Figure 2). All files must be kept within the same directory.
   c. Launch the anaconda powershell prompt and change the directory to where PySpore is located.

```
cd disk:\path to PySpore directory\PySpore
```

   d. Install dependencies by installing the included environment file.

```
(base) conda env create -f pyspore_env.yml
```

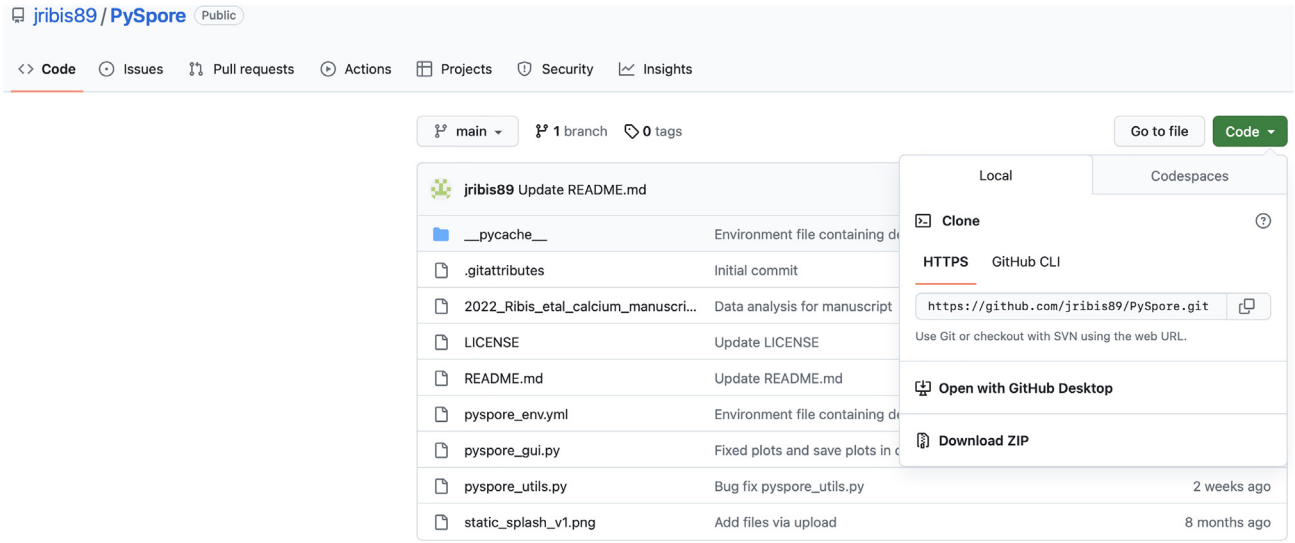

Figure 2. PySpore download page on GitHub

Screenshot of download page. Users download the .zip file and extract to their directory of choice.

e. Activate the environment.

```
(base) conda activate pyspore_env.yml
```

f. Test installation by launching PySpore.

```
(pyspore_env) python "pyspore_gui.py"
```

g. After launching, the user should be greeted by a splash screen followed by a launcher to open the image processing GUI or the data explorer GUI (see below). For a video tutorial, please go to the following link (https://www.youtube.com/watch?v=9ac-tRCYk20).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TopVision Low Melting Point Agarose	Thermo Scientific	FERR0491
Sodium taurocholate	Biosynth International	FS10907
Software and algorithms		
PySpore	This study	https://github.com/jribis89/PySpore
Other		
Gene Frame, 125 µL (1.7 × 2.8 cm)	Thermo Fisher Scientific	AB0578
VWR VistaVision Microscope Slides (Plain)	VWR	16004–422
VWR VistaVision Coverglass (#1.5)	VWR	16004–348
Incubated microscope with phase-contrast optics	Leica Microsystems	Dmi8 inverted microscope stand, incubation enclosure, 1.4 NA plan apochromat phase contrast oil objective, LasX acquisition software, hardware autofocus (Adaptive Focus Control).

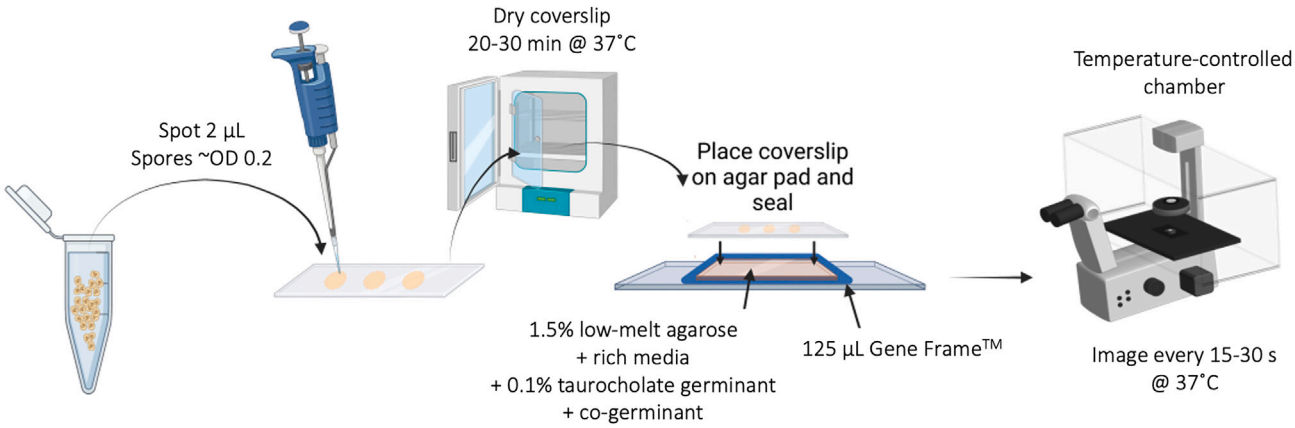

Figure 3. Workflow schematic of single-spore imaging
Created in part with BioRender.com TA refers to the bile acid germinant, taurocholate.[7]

MATERIALS AND EQUIPMENT

3.5% low-melting weight agarose: add 1.75 g into 50 mL mQH_2O in a Pyrex bottle. Bring to a boil in the microwave. Aliquot in 1-mL increments into 1.5-mL microcentrifuge tubes. Heat to 95°C prior to use to melt the agarose prior to use.

10% taurocholate (TA): add 1 g sodium taurocholate into ~9 mL mQH_2O to dissolve. Bring the volume up to 10 mL. Filter-sterilize.

STEP-BY-STEP METHOD DETAILS

Time-lapse imaging of spore germination

Note: If doing the experiment at 37°C, make sure the microscope incubator is turned on at least 5 h prior to the start of the experiment to equilibrate the temperature.

1. Prepare the spore suspension (Figure 3) from a stock of purified spores.

   Note: Spores are purified from sporulating cultures grown on 70:30 agar media after multiple water washes, DNAse treatment, and gradient centrifugation to obtain a >95% pure population of *C. difficile* spores.[10,11]

2. Dilute purified spores to 0.2–0.5 OD600 in a final volume of 50–100 µL depending on how many experiments are planned for that respective prep.
3. Prepare coverslip on which spores will be inoculated.
   a. Clean coverslip (#1.5H thickness) using 70% ethanol followed by deionized water. Wipe with a folded Kimwipe.

   Note: Fold, but do not crumple, the Kimwipe to avoid excessive lint.

   Note: The coverslip size depends on the size of Gene Frame used. For 25 µL Gene Frames, we use a 22 mm × 22 mm coverslip. For 100 µL Gene Frames, we use a 24 × 40 mm coverslip.

   b. Draw a 3 × 2 grid (if using a 100 µL Gene Frame) on a coverslip using permanent marker if imaging multiple strains or preps in the same assay.
   c. Spot 2–3 µL diluted spores onto their respective grid position on the coverslip and let the spots dry at 37°C for 20–30 min or at ~25°C for 30–60 min.

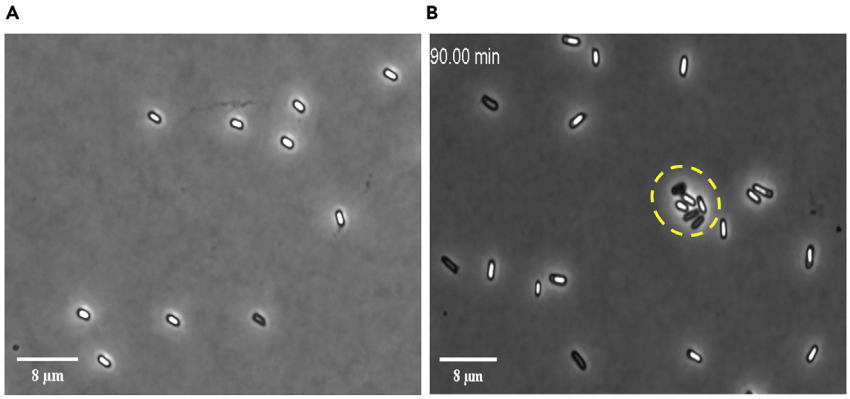

Figure 4. Spore density in movies

(A) Evenly spaced spores at a good density for segmentation.

(B) A clump of spores is outlined in yellow that can decrease the accuracy of the segmentation. Scale bar shows 8 μm.

Note: Ideally, you will have isolated single spores with as few clumps as possible. The spore dilution used should be optimized to minimize clumping (Figure 4).

4. Prepare imaging chamber.
   a. Clean two standard microscope slides with 70% ethanol followed by deionized water.
   b. Remove the solid adhesive backing, keeping the one on the opposite side (with a center hole), of a Gene Frame and adhere to the center of one of the clean slides.

⚠ CRITICAL: Avoid introducing bubbles between the Gene Frame and the slide to create an airtight seal (troubleshooting problem 1).

5. Prepare agarose with germinant.
   a. Make a 3.5% solution of low melting point agarose in deionized water.
   b. Make a 2× concentrated solution of media/buffer for germination containing germinant signals.

Note: For *C. difficile*, BHIS (rich medium)[12] containing 0.2%–0.5% sodium taurocholate is adequate to initiate germination.[1] If using a buffer containing taurocholate, the buffer must include either $CaCl_2$ or glycine as a co-germinant.[1]

6. Prepare agarose pad.
   a. Vortex 1 mL molten agarose and 1 mL 2× media thoroughly, then quickly pipet 500 μL of the solution into the well created by the Gene Frame.

Note: See Wilmaerts et al. for more details about setting up the agarose pad (troubleshooting problem 1).[13]

   b. Quickly cover with another clean microscope slide, as agarose will spill from the sides. Press the top slide down firmly on the Gene Frame.

Note: Gene Frames are gas impermeable, which is a useful feature when studying the outgrowth of germinating spores from anaerobic bacteria. However, for obligate or facultative aerobes, the use of Gene Frames for longer-term imaging studies may alter the outgrowth kinetics measured. Since germination is insensitive to oxygen, the use of Gene Frames will not affect germination kinetics.[14,15]

c. Let solidify at 4°C for 30 min or 25°C for 2–5 h. Ensure that the agarose pad equilibrates to the temperature that the experiment will be conducted (e.g., 25°C or 37°C) before adding the coverslip (troubleshooting problems 1 & 3).

7. Set up image acquisition parameters.
   a. Select a 60–100× high numerical aperture phase-contrast oil immersion objective.
   b. Find a suitable illumination intensity and exposure time.

⚠ CRITICAL: It is important to avoid saturated pixels. Phase-bright spores are so "bright" that their intensity can easily saturate the camera. For guidance optimizing illumination conditions, see the following ref. 16 and 17 (troubleshooting problem 2)

   c. Set the experiment duration optimized for your experiment and the sampling interval (30 s maximum to avoid under-sampling).
8. Inoculate spores onto agarose pad.
   a. Take the top slide off the agarose pad, pull off the top adhesive backing, then let the pad dry for 3–5 min at 25°C.
   b. Take the coverslip with adhered spores and carefully place it on the agar pad, sealing the edges.
   c. Place the slide on the stage insert (holder should accommodate a standard glass slide).
   d. Immediately pick two different XY positions throughout each sample and begin imaging.

CRITICAL: Working quickly is essential to track germination. Ideally, you should start imaging within 1–1.5 min.

Note: Choosing multiple XY positions ensures that even if one of them goes out of focus, the other positions will still allow for useful data to be acquired. However, the use of multiple XY positions can result in changes in focus when the stage moves due to oil dragging. Adding a "wait" command of 100–500 ms should ensure that the oil is spread evenly on the top lens (troubleshooting problems 2 & 3).

Note: It is important to choose positions that are not too crowded and avoids clumps as the segmentation becomes less accurate. An example of an ideal density of spores and one that has clumps is provided in (Figure 4).

Image processing using PySpore

Timing: Varies depending on the size of the dataset

9. Preprocess images (Figure 5).
   a. Crop and export files in tagged image format (.tif).

Note: Although PySpore is robust to images that have some deviation in illumination across the field, it is important to curate the images and crop portions of the image that are out of focus. It may also be necessary to discard movies that drift in and out of focus between frames. Having as flat an agarose pad as possible will minimize these issues (troubleshooting problems 1 & 2).

⚠ CRITICAL: Images must be fed into the software as a stack of .tif files, not individual frames.

   b. In FIJI/ImageJ or image acquisition software, crop out out-of-focus regions from the image and trim the number of frames as needed.

c. Filenames should contain the channel and position of the image. For example: WT_A1 Position1_Crop001_RAW_ch00.tif (the naming convention here refers to the strain (WT); slide region (A1), and given XY position (Position1).

Note: There are no specific requirements for the naming convention, but distinguishing between TIFF stacks analyzed based on strain and position would be helpful.

d. Launch PySpore.
e. Click on the "Launch Image Processing" button within the launcher. At this point, the image processing GUI will pop up (Figure 5).
f. Select "Browse" (Figure 5, yellow oval) from the top of the GUI. Navigate to the directory containing the pre-processed images. The image names will appear in the list box found at the center of the GUI.
g. Load an image by selecting it from the list and clicking the "Load Image" icon (Figure 5, orange rectangle).

Note: At the bottom of the window, a slider will appear, allowing the user to view all images in the stack.

h. Optimize segmentation by clicking "Test Segmentation" (Figure 5, green rectangle). An image showing the spore outlines generated will be displayed.

Note: If spores are poorly segmented and small pieces of debris are highlighted, adjust the threshold to a higher value. Typical values range between 0.05-0.15. As part of the image processing scheme, the image undergoes a black-hat transform to accentuate the dark spore coat along with phase-gray and phase-dark spores, so the thresholds do not reflect the intensities of the input image. Testing the threshold should be done for multiple time points in the movie. Do this by using the frame slider noted in the previous step.

Note: If significant drift is observed between frames, check the box to align the images (Figure 6). The program will do so using phase cross correlation to generate an offset between the current frame and the next and translate the images (shift in x,y) using the calculated offset.

10. Process the images.
    a. Highlight one or more files and select either "Process" or "Batch Process" (Figure 6).

Note: The progress of the analysis is displayed in the command line, and the output contains (1) the resulting segmentation masks and (2) the .csv data containing relevant spore properties quantified.

    b. Enter relevant experimental data associated with each image (Figure 6).
    c. Select the "Save Experiment Data" button to ensure the data is propagated to the output.

Data processing using PySpore

Timing: Varies depending on the size of the dataset and computer hardware

11. Explore PySpore output data.
    a. Plot the data to generate simple interactive plots of spore germination traces displayed in a web browser (Figure 7).

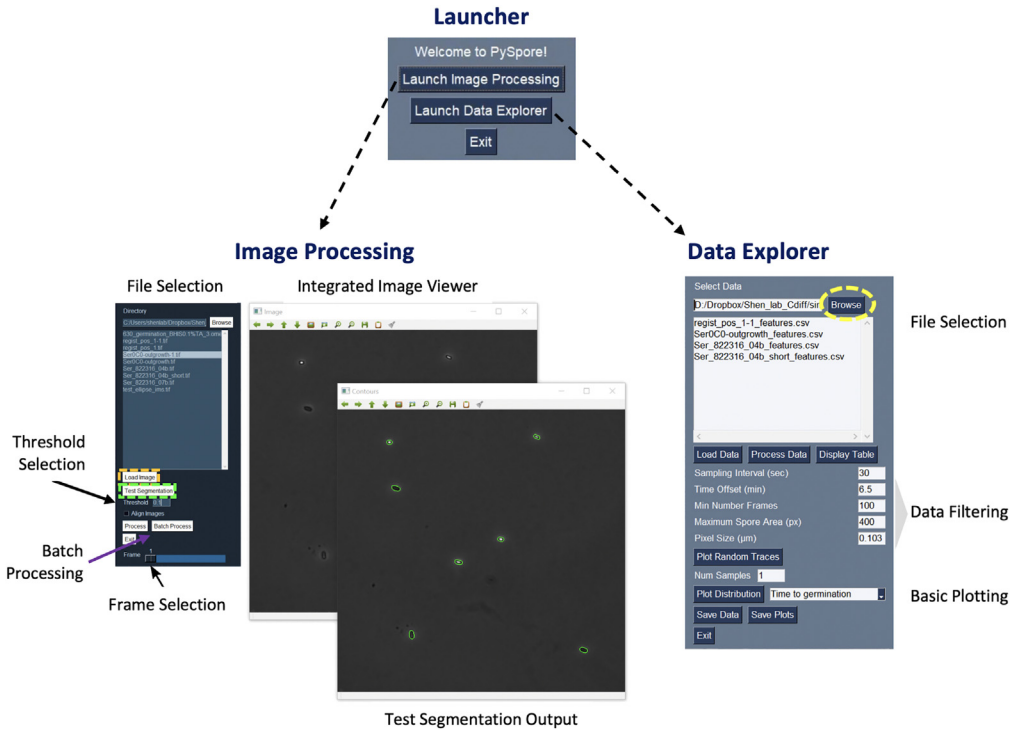

Figure 5. PySpore image processing and data processing GUIs

PySpore image processing and data processing GUIs. Highlighted features are shown including an interactive image viewer, interactive threshold selection to optimize parameters, a selection for whether the images in the TIFF stack should be aligned prior to segmentation to facilitate accurate tracking, a batch processing interface, and simple file selection. A data processing interface is also included which allows the user to plot and explore data, filter data, and join multiple datasets together.

> *Note:*. PySpore also (1) provides functionality to join datasets together, (2) assigns unique identifiers to spores in joined datasets, and (3) allows for easy gating on specific parameters, including spore area to exclude clumps and the number of frames a spore was tracked (Figure 8). In addition, PySpore allows the user to input a time offset (time delay to the start of the acquisition) and correct for the pixel size based on the camera and objective magnification. The combined dataset is compiled in "long form" where rows contain each measurement at a given time and each column represents a different variable that was measured and thus should be amenable to plotting and statistical analysis in software, including GraphPad Prism.

EXPECTED OUTCOMES

PySpore provides a straightforward means for analyzing bacterial spore germination at single-cell resolution from time-lapse phase contrast microscopy datasets. In our experience, PySpore generalizes well to images taken from different systems.

Importantly, PySpore provides significantly more meaningful data than bulk experiments due to its ability to capture and quantify heterogeneity within a population of germinating spores. In addition, the output data from PySpore allows researchers to deconvolve heterogeneity in the time to germination and germination rate, both of which cannot be distinguished in bulk analyses.[1,18]

QUANTIFICATION AND STATISTICAL ANALYSIS

The main output of PySpore is a .csv file containing several quantified spore attributes including area, mean/maximum intensity, xy position, and several others at each timepoint. Refer to the included Jupyter Notebook for a more comprehensive overview of data analysis.

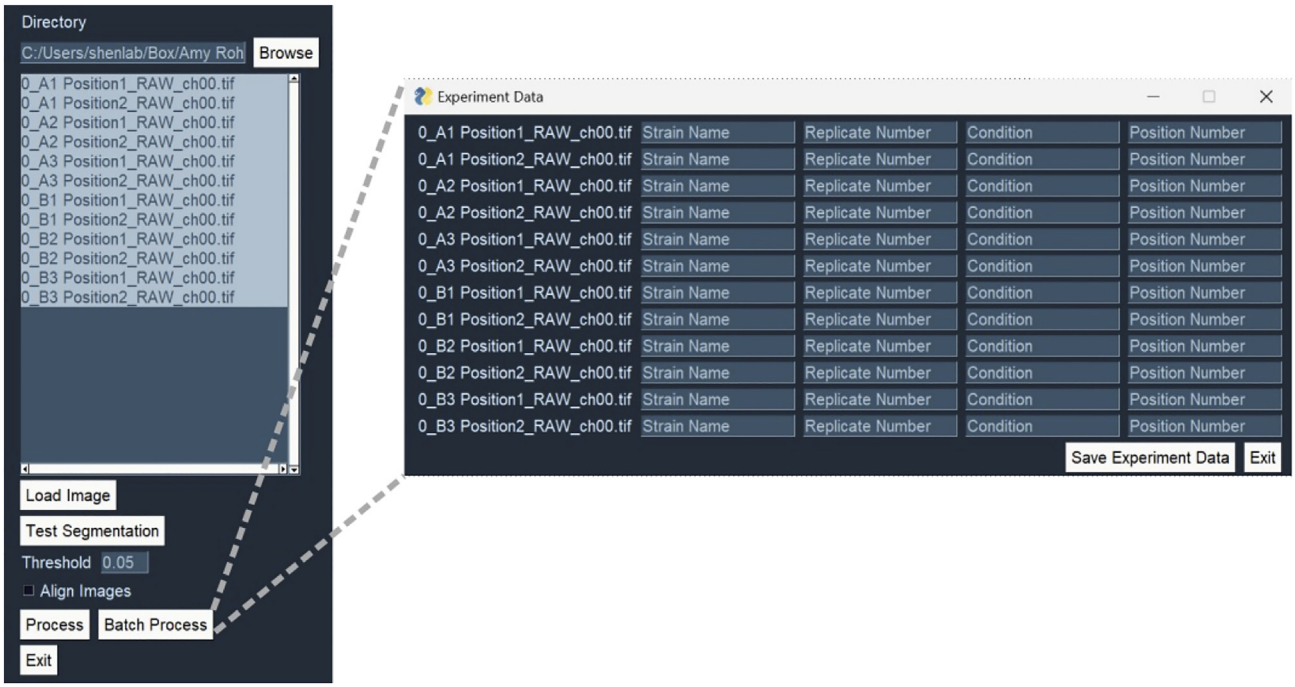

Figure 6. Batch image processing interface

PySpore allows the user to process multiple images after selecting an appropriate threshold. Upon highlighting all images to be processed, the user selects "Batch Process" which will bring up a new window prompting the user to enter relevant data for the experiment. Once the data is added, the user selects "Save Experiment Data", and a popup will appear indicating that the operation was successful. To start the batch analysis, the user presses the "Exit" button.

(https://github.com/jribis89/PySpore/blob/main/2022_Ribis_etal_calcium_manuscript.ipynb).

Since the intensity measurements can be noisy, traces with high noise are removed from the data. To accomplish this, the coefficient of variation (CV) is calculated at different windows in the data. If several regions of high variation are measured above a user-defined threshold, the data is excluded from downstream analysis.

Germination rate is measured by fitting a spline to the data rather than trying to fit a single function (Figure 9).[1] After a spline is fitted, the maximum rate is determined by taking the first derivative of the spline at each timepoint. Since there is a decrease in the intensity of the spores during germination, the maximum rate corresponds to the minimum value of the derivative evaluated at all timepoints. To simplify the interpretation of these data, we display the rate as an absolute value, so the higher the number, the faster the rate of germination. The germination time is defined as the time point when the maximal germination rate is observed.

LIMITATIONS

Since PySpore does not leverage machine or deep-learning algorithms for segmentation, separating spores in tight clumps will typically fail. Because of this, the input images require spores to be well separated from one another.

PySpore currently lacks the functionality to quantify fluorescent signal, but the modularity of the code should allow the user to add this should they wish.

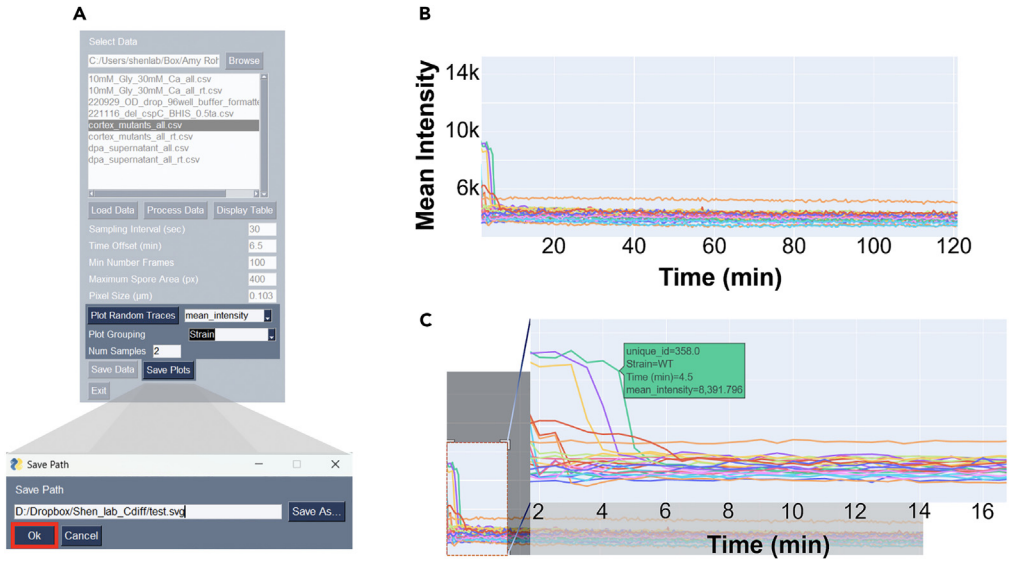

Figure 7. Interactive single-spore plots of the data integrated into the data explorer

(A) Plotting functionality with dropdowns for plotting parameters and for plot row grouping. Users enter the number of spores they want plotted from each position in the "Num Samples" box and click "plot random traces" to generate plots.

(B) Plots are generated using the Plotly Express Python library and are displayed in html format in a web browser. Selecting save plots will allow the user to save the plot locally as a .svg by default, though a different extension including .png or .tif can be denoted.

(C) Mousing over individual traces shows data for an individual spore at any timepoint. Portions of the plot can be highlighted and viewed in higher detail.

While PySpore cannot currently be used to analyze the properties of spores in an image (e.g., length, width, ellipsoidicity, etc.), the program can be modified to provide these properties if the images are made into a two-frame "movie."

TROUBLESHOOTING

Problem 1

Image drift is observed between frames.

Potential solution

- Ensure that the edges of the frame surrounding the agar pad and the coverslip are sealed well, e.g., by running a closed Sharpie pen around the edges (Steps 2,4).
- Ensure that the sample and scope are at the same temperature (e.g., 37°C). Keep all other materials at temperature (e.g., 37°C, agarose pad, coverslip). (Step 1)
- Ensure that the agarose pads are not too dry or wet, since this can lead to more image drift (Step 6).
- Press lightly on the coverslip before starting the experiment.
- Drift can often be corrected by the program using the built-in image registration/drift correction. The user can select this option in the image processing GUI by selecting "Align Images" (Step 7).

Problem 2

High noise in data, with germination traces exhibiting erratic changes in intensity.

Potential solution

- Set up Köhler illumination on the phase contrast microscope, to get the best phase-contrast images by ensuring that the illumination is uniform across a field-of-view. Ideally, this should be done on a regular basis (e.g., before each experiment).

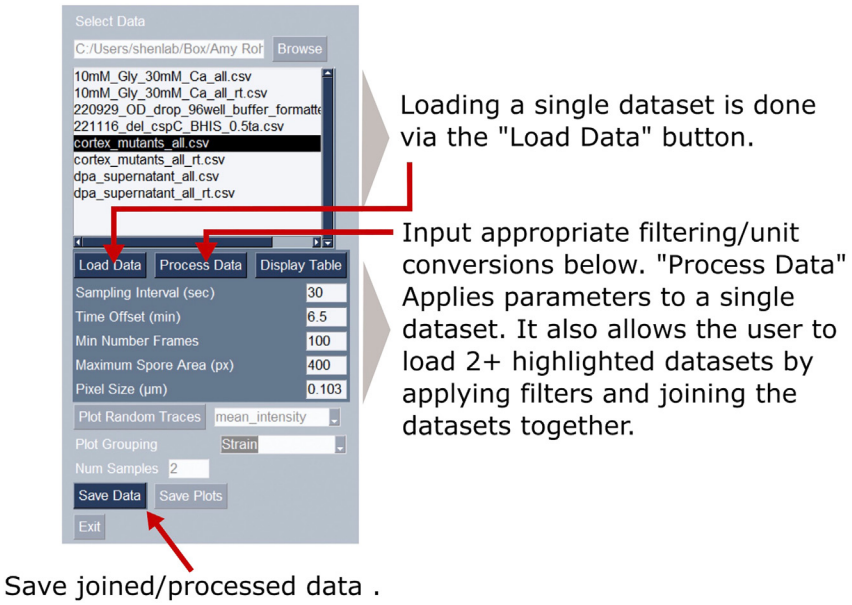

Figure 8. Data processing fields in data explorer GUI

GUI allows users to join and process datasets with multiple filtering parameters, pixel conversion, and time conversion.

- Take steps to mitigate vibration during the imaging session, i.e., avoid imaging around freezers and refrigerators and use a vibration isolation table; can also leave the shutter open during acquisition to minimize blurring and minimize vibrations caused by the shutter opening.
- Ensure that the image histogram is within range (Step 5).

Problem 3

Loss of focus between frames.

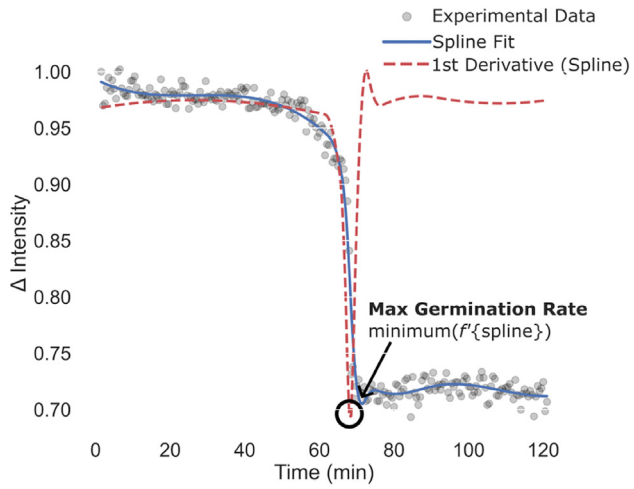

Figure 9. Spline determination of germination rate and germination time

Plots showing the change in intensity of a germinating spore. The points reflect raw data, with the solid blue line showing the fit of a 1D smoothing spline. The dashed red line shows the first derivative of the fitted spline evaluated at each time point, and the minimum is circled to highlight the point where the maximal germination rate is reached, which is defined as the time to germination. Figure was modified from Ribis et al. (2023).[1]

Potential solution

- Use an imaging system with autofocus maintenance device and a compatible objective. Make sure the objective is clean, as this can affect the performance of the hardware-based focus maintenance.
- Avoid external sources of IR light that could interfere with hardware autofocus.
- Ensure that the agarose pad has temperature equilibrated before imaging (Step 4)
- Using pads that are too fresh, i.e., have not solidified for longer periods of time, can lead to a loss of focus over time.[13] (Step 4)
- Dense populations of spores may affect the performance of the focus maintenance device.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Aimee Shen (aimee.shen@tufts.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code generated during this study is available at https://github.com/jribis89/PySpore.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.W.R. and A.S.; methodology, J.W.R.; software, J.W.R.; formal analysis, J.W.R.; investigation, J.W.R.; writing – original draft, J.W.R.; writing – review & editing, J.W.R. and A.S.; resources, J.W.R.; visualization, J.W.R. and A.S.; supervision, A.S.; funding acquisition, A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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